

# Interaction with the inositol 1,4,5-trisphosphate receptor promotes $\text{Ca}^{2+}$ sequestration in permeabilised insulin-secreting cells

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Received 7 June 1991

Electropermeabilised insulin-secreting RINm5F cells sequestered  $\text{Ca}^{2+}$ , resulting in a steady-state level of the ambient free  $\text{Ca}^{2+}$  concentration corresponding to  $723 \pm 127$  nM (mean  $\pm$  SEM,  $n=10$ ), as monitored by a  $\text{Ca}^{2+}$ -selective minielectrode. Inositol 1,4,5-trisphosphate ( $\text{Ins}(1,4,5)\text{P}_3$ ) promoted a rapid and pronounced release of  $\text{Ca}^{2+}$ . This  $\text{Ca}^{2+}$  was resequenced and a new steady-state  $\text{Ca}^{2+}$  level was attained, which was always lower ( $460 \pm 102$  nM,  $n=10$ ,  $P<0.001$ ) than the steady-state  $\text{Ca}^{2+}$  level maintained before the addition of  $\text{Ins}(1,4,5)\text{P}_3$ . Whereas the initial reuptake of  $\text{Ca}^{2+}$  subsequent to  $\text{Ins}(1,4,5)\text{P}_3$  stimulation was relatively slow, the later part of reuptake was fast as compared to the reuptake phases of a pulse addition of extraneous  $\text{Ca}^{2+}$ . In the latter case the uptake of  $\text{Ca}^{2+}$  resulted in a steady-state level similar to that found in the absence of  $\text{Ins}(1,4,5)\text{P}_3$ . Addition of  $\text{Ins}(1,4,5)\text{P}_3$  under this condition resulted in a further  $\text{Ca}^{2+}$  uptake and thus a lower steady-state  $\text{Ca}^{2+}$  level. Heparin, which binds to the  $\text{Ins}(1,4,5)\text{P}_3$  receptor, also lowered the steady-state free  $\text{Ca}^{2+}$  concentration. In contrast to  $\text{Ins}(1,4,5)\text{P}_3$ , inositol 1,3,4,5-tetrakisphosphate was without effect on  $\text{Ca}^{2+}$  sequestration. These findings are consistent with the presence of a high-affinity  $\text{Ins}(1,4,5)\text{P}_3$  receptor promoting continuous release of  $\text{Ca}^{2+}$  under basal conditions and/or the  $\text{Ins}(1,4,5)\text{P}_3$  receptor being actively involved in  $\text{Ca}^{2+}$  sequestration.

Inositol 1,4,5-trisphosphate; Inositol 1,4,5-trisphosphate receptor; Intracellular  $\text{Ca}^{2+}$ -transport; Insulin-secreting cell

## 1. INTRODUCTION

Not only inositol 1,4,5-trisphosphate ( $\text{Ins}(1,4,5)\text{P}_3$ ) but also inositol 1,3,4,5-tetrakisphosphate ( $\text{Ins}(1,3,4,5)\text{P}_4$ ), one of its metabolites, is known to be involved in the generation of intracellular  $\text{Ca}^{2+}$  signals [1–3].  $\text{Ins}(1,4,5)\text{P}_3$  rapidly mobilizes intracellular  $\text{Ca}^{2+}$  stores in a wide variety of permeabilized cells, including insulin secreting RINm5F cells [4] and normal pancreatic  $\beta$ -cells [5]. The resulting rise in  $\text{Ca}^{2+}$  is short-lived and the ambient free  $\text{Ca}^{2+}$  concentration is eventually returned to basal levels reflecting reuptake into intracellular  $\text{Ca}^{2+}$  pools. Regulation of  $\text{Ca}^{2+}$  reuptake into intracellular  $\text{Ca}^{2+}$  pools following  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $\text{Ca}^{2+}$  release is poorly understood.  $\text{Ins}(1,3,4,5)\text{P}_4$  has been suggested to act in concert with  $\text{Ins}(1,4,5)\text{P}_3$  in the mobilization of intracellular  $\text{Ca}^{2+}$  [2,3] and it has also been suggested that the tetrakisphosphate induces  $\text{Ca}^{2+}$  sequestration [6].

Using electropermeabilised insulin-secreting RINm5F cells and  $\text{Ca}^{2+}$  selective minielectrodes, we have investigated the reuptake of  $\text{Ca}^{2+}$  following its release by  $\text{Ins}(1,4,5)\text{P}_3$ . We now demonstrate that interaction with the  $\text{Ins}(1,4,5)\text{P}_3$  receptor, in addition to

releasing  $\text{Ca}^{2+}$ , also promotes the reuptake of this ion, resulting in a lowered ambient steady-state  $\text{Ca}^{2+}$  concentration.

## 2. MATERIALS AND METHODS

Clonal insulin-secreting RINm5F cells were maintained in culture in RPMI 1640 medium supplemented with 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100  $\mu\text{g}/\text{ml}$ ), all from Flow Laboratories (Scotland).

$\text{Ins}(1,4,5)\text{P}_3$  (potassium salt) was purchased from Sigma (St. Louis, USA). HPLC pure  $\text{Ins}(1,3,4,5)\text{P}_4$  and  $\text{Ins}(2,4,5)\text{P}_3$  were generous gifts from Dr R.F. Irvine, Cambridge, UK. Calcium ionophore cocktail, containing neutral carrier ETH 1001 was from Fluka. All other chemicals were of highly purified grade and were either from Sigma or Merck.

Cells were detached from culture flasks using Trypsin-EDTA. They were then washed twice with culture medium and twice with a cold nominally  $\text{Ca}^{2+}$  free buffer, containing 110 mM KCl, 10 mM NaCl, 2 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{MgCl}_2$ , 0.5 mg/ml bovine serum albumin and 25 mM HEPES, pH 7.0 (adjusted with KOH). After washing the cells were permeabilised by exposure to high-voltage discharges (six pulses of 3.2 kV/cm). This treatment resulted in more than 90% permeabilised cells, as verified by Trypan blue uptake. After permeabilisation, cells were centrifuged and the pellet was kept on ice until use.

8  $\mu\text{l}$  of cell pellet was added to a plexiglass chamber containing 52  $\mu\text{l}$  of incubation buffer. The incubation buffer was supplemented with 2 mM MgATP and an ATP regenerating system, consisting of 10 mM phosphocreatine and 20 U/ml of creatine kinase. The incubation buffer also contained mitochondrial inhibitors consisting of 0.2  $\mu\text{M}$  antimycin and 1  $\mu\text{g}/\text{ml}$  of oligomycin. Experiments were carried out at room temperature and the cell suspension was stirred continuously, using a small magnetic bar. Additions were made from 100–200 times concentrated solutions. Changes in the ambient free  $\text{Ca}^{2+}$  concentra-

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tion were recorded using a  $\text{Ca}^{2+}$ -selective minielectrode constructed and calibrated essentially as described by Tsien and Rink [7]. Calibration of the electrode was done at the beginning and at the end of each experiment. All data on  $\text{Ca}^{2+}$  concentrations are given as mean values  $\pm$  SEM and statistical significances were judged by Student's *t*-test for paired data.

### 3. RESULTS AND DISCUSSION

Addition of permeabilised RINm5F cells ( $4.7 \times 10^7$  cells/ml) resulted in a lowering of the ambient free  $\text{Ca}^{2+}$ -concentration to a steady-state level of  $723 \pm 127$  nM, from the initial  $\text{Ca}^{2+}$  level of  $4.5 \pm 0.18$   $\mu\text{M}$  ( $n = 10$ ). Stimulation with 5  $\mu\text{M}$  Ins(1,4,5) $\text{P}_3$  induced a prompt release of  $\text{Ca}^{2+}$  reaching a value of  $2.72 \pm 0.16$   $\mu\text{M}$  ( $n = 10$ ), which was slowly taken up again (Fig. 1A). The new steady-state  $\text{Ca}^{2+}$  level obtained was lower ( $P < 0.001$ ) than prior to stimulation with Ins(1,4,5) $\text{P}_3$  and corresponded to  $460 \pm 102$  nM ( $n = 10$ ).

When a pulse of  $\text{CaCl}_2$  (0.6 nmol) was added to the cells, the initial uptake of the ion was rapid but the terminal part of the uptake phase was slow and the  $\text{Ca}^{2+}$  concentration was maintained at a slightly elevated level, during the period of observation (Fig. 1B). Ins(1,4,5) $\text{P}_3$  added under these conditions induced a normal release of  $\text{Ca}^{2+}$ . In this case the initial phase of reuptake of  $\text{Ca}^{2+}$  was relatively slow, as compared to the initial rapid uptake following an extraneous  $\text{Ca}^{2+}$  pulse. However, the later part of the reuptake phase was rapid, reaching a steady-state level that was lower than that prior to the addition of  $\text{CaCl}_2$  or Ins(1,4,5) $\text{P}_3$ . Previous studies that have addressed the effect of Ins(1,4,5) $\text{P}_3$  on  $\text{Ca}^{2+}$  handling in permeabilised cells

have not discussed the lowering effect of the trisphosphate on the steady-state  $\text{Ca}^{2+}$  level [8–13]. This effect is easy to miss if Ins(1,4,5) $\text{P}_3$  is added during continuing  $\text{Ca}^{2+}$ -uptake and if enough time is not allowed before and after the actual additions [8–12]. In neoplastic rat liver epithelial cells Ins(1,3,4,5) $\text{P}_4$  has been found to promote sequestration of pulse additions of  $\text{Ca}^{2+}$  or  $\text{Ca}^{2+}$  released by Ins(2,4,5) $\text{P}_3$  [6]. However, under these conditions 2  $\mu\text{M}$  Ins(1,3,4,5) $\text{P}_4$  did not lower the steady-state free  $\text{Ca}^{2+}$  concentration and the intracellular  $\text{Ca}^{2+}$  pools had to be saturated prior to the addition of Ins(1,3,4,5) $\text{P}_4$ . We were unable to demonstrate any lowering effect of 2.5  $\mu\text{M}$  Ins(1,3,4,5) $\text{P}_4$  on the steady-state  $\text{Ca}^{2+}$  level (Fig. 2). Rather, at a concentration of 5  $\mu\text{M}$ , Ins(1,3,4,5) $\text{P}_4$  induced a small increase in  $\text{Ca}^{2+}$  (data not shown). It is therefore unlikely, that the increased  $\text{Ca}^{2+}$  sequestration following Ins(1,4,5) $\text{P}_3$ -induced  $\text{Ca}^{2+}$  release is mediated through metabolism of Ins(1,4,5) $\text{P}_3$  to Ins(1,3,4,5) $\text{P}_4$ .

In the presence of heparin (200  $\mu\text{g}/\text{ml}$ ), which binds to the Ins(1,4,5) $\text{P}_3$  receptor [14] and blocks the trisphosphate-mediated release of  $\text{Ca}^{2+}$  [15–17], Ins(1,4,5) $\text{P}_3$  failed to induce a rise in the  $\text{Ca}^{2+}$  concentration, but the lowering effect on the steady-state  $\text{Ca}^{2+}$  concentration was still evident, starting approximately a minute after addition of Ins(1,4,5) $\text{P}_3$  (Fig. 3A). As evident from Fig. 3B, heparin also blocked the  $\text{Ca}^{2+}$  release evoked by Ins(2,4,5) $\text{P}_3$ , a non-metabolisable analogue of Ins(1,4,5) $\text{P}_3$ , resulting in a similar lowering in  $\text{Ca}^{2+}$  as that obtained in the presence of heparin plus Ins(1,4,5) $\text{P}_3$ . Interestingly, it was observed that heparin alone also lowered the steady-state  $\text{Ca}^{2+}$  level (Fig. 4). That heparin by itself may cause increased sequestration of  $\text{Ca}^{2+}$  is apparent from at least one other study [17]. The same study also demonstrated that heparin-induced reuptake of Ins(1,4,5) $\text{P}_3$ -released  $\text{Ca}^{2+}$  was extremely rapid. Noteworthy is that the effects of Ins(1,4,5) $\text{P}_3$  and heparin on  $\text{Ca}^{2+}$  sequestration were not additive (cf. Figs. 1, 3 and 4), suggesting that these agonists act through the same mechanism.

The mechanism(s) by which Ins(1,4,5) $\text{P}_3$  and heparin induce sequestration of  $\text{Ca}^{2+}$  can only be speculated upon at this stage. One possibility is the existence of high-affinity Ins(1,4,5) $\text{P}_3$  receptors. Under basal condi-

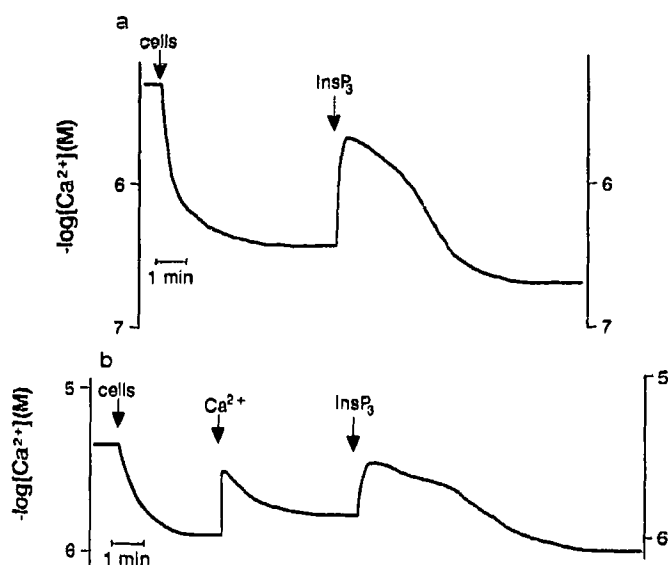


Fig. 1. Effect of Ins(1,4,5) $\text{P}_3$  on the steady-state free  $\text{Ca}^{2+}$  concentration. The figure shows  $\text{Ca}^{2+}$ -electrode traces obtained under conditions described in materials and methods section. A. At the point indicated Ins(1,4,5) $\text{P}_3$  (5  $\mu\text{M}$ , final concentration) was added. The trace is typical of 6 independent experiments. B. At points indicated  $\text{CaCl}_2$  (0.6 nmol) or Ins(1,4,5) $\text{P}_3$  (5  $\mu\text{M}$ , final concentration) was added. The trace is representative of 4 different experiments.

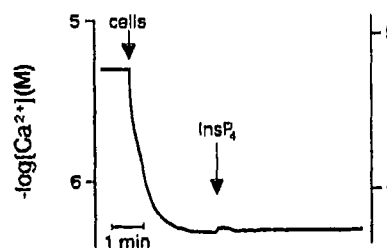


Fig. 2. Effect of Ins(1,3,4,5) $\text{P}_4$  on the steady-state free  $\text{Ca}^{2+}$  concentration. At the point indicated Ins(1,3,4,5) $\text{P}_4$  (2.5  $\mu\text{M}$ , final concentration) was added. The trace is typical of three different experiments.

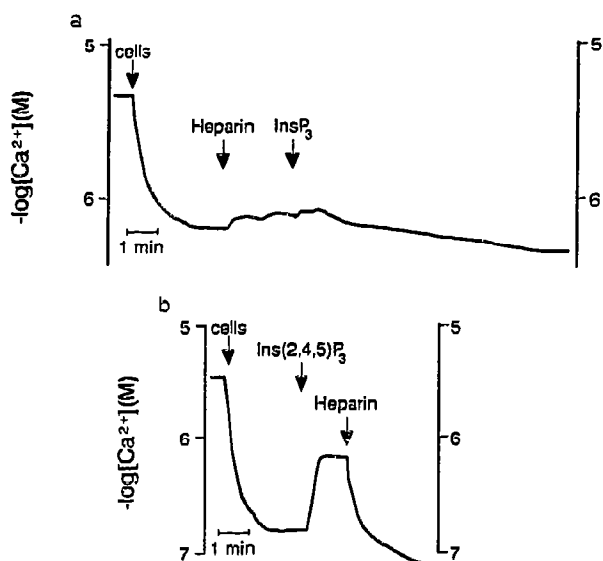


Fig. 3. Effect of  $\text{Ins}(1,4,5)\text{P}_3$  in the presence of heparin, on the steady-state free  $\text{Ca}^{2+}$  concentration (A). Heparin ( $200 \mu\text{g}/\text{ml}$ ) and  $\text{Ins}(1,4,5)\text{P}_3$  ( $5 \mu\text{M}$ ) were added as indicated. The trace is representative of three different experiments. B. Effect of adding heparin after  $\text{Ins}(2,4,5)\text{P}_3$ -induced  $\text{Ca}^{2+}$  release. As indicated  $\text{Ins}(2,4,5)\text{P}_3$  ( $5 \mu\text{M}$ ) or heparin ( $200 \mu\text{g}/\text{ml}$ ) were added. The trace is representative of three different experiments.

tions low levels of  $\text{Ins}(1,4,5)\text{P}_3$  [11] mediate continuous mobilisation of  $\text{Ca}^{2+}$  from endoplasmic reticulum, thus balancing the uptake of the ion. Following exposure to high concentrations of  $\text{Ins}(1,4,5)\text{P}_3$  these receptors may become down-regulated and therefore  $\text{Ca}^{2+}$  uptake is not counteracted, resulting in a more pronounced buffering of  $\text{Ca}^{2+}$ . By binding to these high-affinity  $\text{Ins}(1,4,5)\text{P}_3$  receptors heparin will block the  $\text{Ca}^{2+}$  release pathway operating under basal conditions, the net effect also in this case being an increased uptake of  $\text{Ca}^{2+}$ . Prentki et al. proposed a role for basal  $\text{Ins}(1,4,5)\text{P}_3$  in regulating  $\text{Ca}^{2+}$  cycling across endoplasmic reticulum [11] and it appears that various cells indeed contain low levels of  $\text{Ins}(1,4,5)\text{P}_3$  even under basal conditions [18]. Hence, the continuous presence of low concentrations of  $\text{Ins}(1,4,5)\text{P}_3$  will enable continuous activation of the high-affinity  $\text{Ins}(1,4,5)\text{P}_3$  receptors. Spät et al. described a type of high-affinity  $\text{Ins}(1,4,5)\text{P}_3$

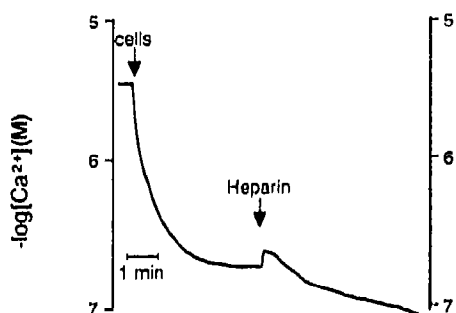


Fig. 4. The effect of heparin on the steady-state free  $\text{Ca}^{2+}$  concentration. Heparin ( $200 \mu\text{g}/\text{ml}$ ) was added as indicated. The trace is typical of three different experiments.

receptor of unknown functional significance in neutrophils [19]. Whether such receptors also exist in RINm5F cells merits further investigations.

Another possibility whereby  $\text{Ins}(1,4,5)\text{P}_3$  and heparin promote increased  $\text{Ca}^{2+}$  sequestration may be through the activation of receptors distinct from the traditional  $\text{Ins}(1,4,5)\text{P}_3$  receptors. In this case heparin, by being a structural analogue of  $\text{Ins}(1,4,5)\text{P}_3$ , may actually promote  $\text{Ca}^{2+}$  uptake by acting like a partial agonist.

We have now demonstrated that the presence of  $\text{Ins}(1,4,5)\text{P}_3$  not only leads to  $\text{Ca}^{2+}$  mobilisation but also a more effective  $\text{Ca}^{2+}$  buffering. Hence, under physiological conditions the trisphosphate may induce sequential both increase and decrease in cytoplasmic free  $\text{Ca}^{2+}$ , enabling complex regulation of intracellular processes dependent on  $\text{Ca}^{2+}$ .

**Acknowledgements:** Financial support was obtained from the Swedish Medical Research Council (19x-00034), the Bank of Sweden Tercentenary Foundation, the Swedish Diabetes Association, the Nordic Insulin Foundation, the Swedish Hoechst Diabetes Research Foundation, the Syskonen Svenssons Foundation, Magnus Bergvalls Foundation, Lars Hiertas Memorial Foundation, Aage and Louis-Hansens Memorial Foundation, NOVO Industry, Farmitalia Carlo-Erba, Fredrik and Ingrid Thuring's Foundation, Tore Nilsons Foundation for Medical Research, the Torsten and Ragnar Söderbergs Foundations, the Swedish Society of Medicine and Funds of the Karolinska Institute.

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